

AD _____

Award Number: DAMD17-03-1-0121

TITLE: The Role of Akt in Androgen-Independent Progression of
Human Prostate Cancer

PRINCIPAL INVESTIGATOR: Benyi Li, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Kansas Medical Center
Kansas City, KS 66160-7114

REPORT DATE: February 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20040621 013

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE February 2004	3. REPORT TYPE AND DATES COVERED Annual (1 Feb 2003 - 31 Jan 2004)	
4. TITLE AND SUBTITLE The Role of Akt in Androgen-Independent Progression of Human Prostate Cancer			5. FUNDING NUMBERS DAMD17-03-1-0121	
6. AUTHOR(S) Benyi Li, M.D., Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Kansas Medical Center Kansas City, KS 66160-7114 E-Mail: bli@kumc.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Original contains color plates: ALL DTIC reproductions will be in black and white				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) To define the causative role of the active Akt in androgen-independent progression of prostate cancer, an inducible Akt (iAKT) is used in this proposal. In this first-year period, androgen-dependent human prostate cancer LNCaP cells were transfected with vectors carrying iAKT system or the control vectors (empty and kinase-dead mutant iAKTkm) to establish stable sublines. We established multiple LNCaP sublines expressing the iAKT system construct, as well as the control vectors. We demonstrated that the established LNCaP.iAKT cells showed a promising ability that the iAKT can be activated after the cells were treated by the dimerization inducer AP22783, and the activated iAKT (F _{pk} 3-ΔphAKT) is functionally capable to perform its physiological task, phosphorylating the downstream target GSK-3. In the next year or step, we will use those LNCaP subline cells to determine whether CID-mediated activation of iAKT promotes survival/proliferation of prostate cancer LNCaP cells after androgen withdrawal.				
14. SUBJECT TERMS Prostate Cancer, Akt/Protein Kinase B; Chemically induced dimerization LNCaP cell line; Cell Culture				15. NUMBER OF PAGES 20
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover.....	
SF 298.....	
Table of Contents.....	
Introduction.....	1
Body.....	1
Key Research Accomplishments.....	4
Reportable Outcomes.....	4
Conclusions.....	4
References.....	4
Appendices.....	5

Annual Report for the Project DAMD17-03-1-0121

February 2004

Project Title:

The Role of Akt in Androgen-independent Progression of Human Prostate Cancer

The PI: Benyi Li, MD/PhD
University of Kansas Medical Center
Kansas City, KS 66160

Introduction

Prostate cancer is the second most frequently diagnosed cancer in men after skin cancer in United States (1). While digital rectal exams and early prostate specific antigen screening have led to earlier detection and diagnosis, the number of new cases continues to rise and present a major worldwide health threat. Medical treatment for metastatic prostate cancer has relied heavily on androgen ablation (2). However, there has been a growing appreciation that most patients treated by androgen ablation ultimately relapse to more aggressive androgen-independent (also called hormone-refractory) prostate cancer with no means to cure (3). Although Akt has been shown to be associated with prostate cancer progression (4-9), determining the causative role of this specific kinase in initiating or maintaining the androgen-independent progression of prostate cancer has not been established. One major limitation is the lack of a well-controlled inducible system to study Akt involvement in prostate cancer. Recently, we have developed a novel inducible Akt (iAKT) system based on chemically induced dimerization (CID) approach. This system allows for selective activation of Akt in a physiological setting. This CID-mediated activation of iAKT is the major innovative feature of this proposal (*Appendix I*). We propose that active Akt plays a causative role in androgen-independent progression of human prostate cancer via protecting cell death and enhancing cell proliferation after androgen withdrawal. *Initially*, androgen-dependent human prostate cancer LNCaP cells will be transfected with vectors carrying iAKT system or control vectors (empty and kinase-dead mutant iAKTkm) to establish stable sublines. *Next*, the sublines will be used to determine whether CID-mediated activation of iAKT can lead to survival or proliferation after androgen withdrawal. *Finally*, LNCaP tumor will be established subcutaneously in male nude mice by inoculating LNCaP subline cells stably expressing iAKT or kinase-dead mutant iAKTkm. After castration or sham-operation, the animals will receive AP22783 injection to induce Akt activation. Tumor growth will be monitored to determine if CID-mediated activation of iAKT promotes androgen-independent LNCaP tumor growth.

Report Body

In this section, we will describe the current status and results obtained from the experiments outlined in the approved Statement of Work, which is listed below:

- Task 1.** (a). Develop prostate cancer LNCaP cell sublines stably expressing iAKT (Months 1-7).
(b). Determine whether CID-mediated membrane targeting of iAKT results in iAKT phosphorylation and activation in LNCaP cells (Months 8-12).

1. Establishment of stable-expressing iAKT cell lines, LNCaP.iAKT.

In the bicistronic vector pBJ5-Neo-iAKT, the two components of the iAKT system were linked via a poliovirus internal ribosome entry sequence (Fig 1, also please see details in *Appendix I*). Therefore, we used this vector for delivering the inducible Akt system in this project. Further, a kinase-dead mutant of this inducible Akt was generated by site directed mutagenesis (QuickChange[®] Site-Directed Mutagenesis Kit, Cat#200519, Stratagene) of protein kinase-conserved lysine 179 (K179M) within the catalytic domain, which produces an inactive form of the kinase. This attenuated construct was designated as iAKTkm and used as negative control to distinguish signaling initiated by the active iAKT from signaling initiated by other signaling molecules (e. g. kinases, phosphatases, and others) that could weakly associate with iAKT.

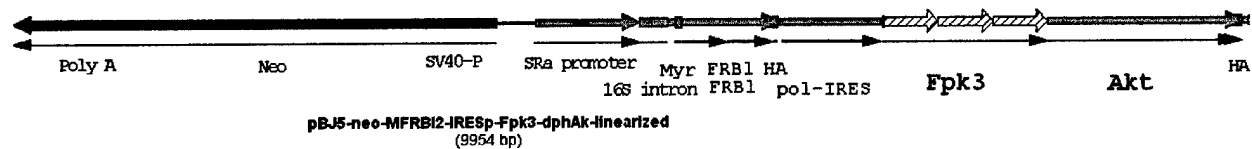


Fig. 1. Schematic representation of the linearized pBJ5-Neo-iAKT plasmid construct

Human prostate cancer cell line LNCaP was used to establish stable sublines for iAKT expression. Linearized pBJ5-Neo-iAKT (Fig. 1) or pBJ5-Neo-iAKTkm plasmids were transfected into the cells with Cytofectene (BioRad). G418-resistant (i.e. growing in 0.5 mg/ml G418) cells were selected in bulk culture, and individual clones were isolated by limiting dilution. The protein levels of the iAKT system (FRB_{L2}, 29kD and F_{pk3}.ΔPHAkt, 91kD) were determined by Western blot using monoclonal antibody HA.11 (BABCO) against the C-terminal epitope (see Fig. 1). We chose the LNCaP.iAKT1 subline (as shown in Fig 2, lane 1) for future experiments. In addition, other three LNCaP

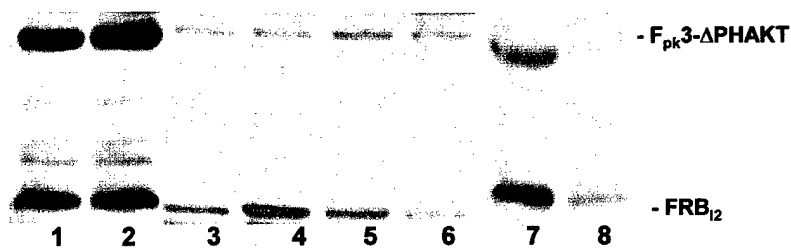


Fig. 2. Multiple clones of LNCaP sublines expressing the iAKT system were established. LNCaP cells were transfected with linearized plasmids pBJ5-Neo-iAKT (lane 1-6) or its kinase-dead mutant pBJ5-Neo-iAKTkm (lane 7-8) using Cytofectene (BioRad). Stable colonies were selected in G418-containing media. The protein expression level of the two compartments (F_{pk3}-ΔphAKT and FRB_{L2}) of the iAKT system was evaluated using Western blotting assay with an anti-HA antibody (HA.11 clone).

sublines were established by transfecting the cells with empty vector pBJ5-Neo (data not shown since no exogenous gene is expressed in Western blot).

2. Determination of CID-mediated Akt activation in LNCaP.iAKT1 cells

Akt is activated after phosphorylation at two sites, T³⁰⁸ and S⁴⁷³, in which S⁴⁷³ phosphorylation is more critical to its full activation (10-11). Thus, we tested whether CID treatment can induce iAkt (F_{pk}3-ΔPHAkt) phosphorylation at S⁴⁷³ site. As shown in Fig. 2, CID addition mediated iAkt phosphorylation in a time- and dose-dependent manner in the stable subline LNCaP.iAKT1 cells.

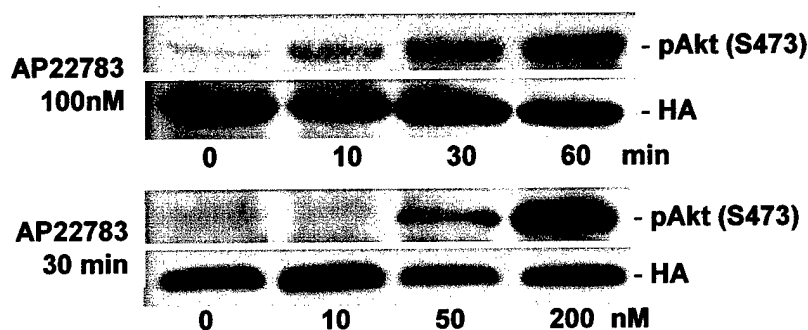


Fig. 3. CID-mediated dose- and time-dependent phosphorylation of the iAKT. LNCaP.iAKT1 cells were serum-starved for 24 hrs and then treated with AP22783 at the doses and for the time period as indicated. After immunoprecipitation with anti-HA antibody (HA.11), Akt phosphorylation at S⁴⁷³ (F_{pk}3-ΔphAKT) was detected by phospho-specific antibodies against Serine 473 of Akt. Anti-HA blots served as loading control.

3. Determine CID-mediated iAKT activation/kinase activity in the stable cell line.

To measure the induction of Akt enzymatic activity, LNCaP.iAKT1 cells were serum starved for 24 h and thereafter treated with AP22783 or serum for 30 min. Chimeric F_{pk}3-ΔphAkt kinase was immunoprecipitated with the anti-HA antibody and Akt kinase activity was measured using an *in vitro* kinase assay that uses a GSK-3α/β 'crosstide' as a substrate. The level of phosphorylated GSK-3 crosstides was determined by immunoblotting with phospho-specific antibody against GSK3-α²¹/β⁹ (detailed in *Appendix I*). As shown in Figure 4, AP22783 treatment resulted in dose- and time-dependent GSK-3 phosphorylation in this assay, indicating that the iAKT was activated after CID-mediated membrane targeting.

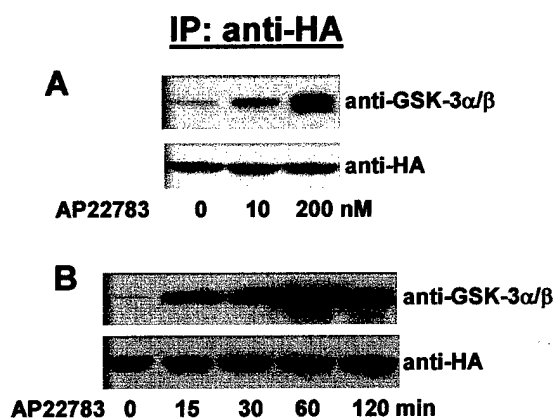


Fig. 4. CID-mediated iAKT activation. LNCaP.iAKT1 cells were serum-starved for 24 h followed by treatment with AP22783 at the doses or for the time period as indicated. The iAKT was immunoprecipitated from cell lysates using anti-HA antibody and incubated with the GSK-3 'crosstide' as a substrate. Phosphorylated GSK-3 crosstides were quantified by SDS-PAGE separation and immunoblotting with a phospho-specific antibody against GSK-3α²¹/β⁹. Anti-HA blot served as loading control.

Key Research Accomplishments

For the first year of this project according to the proved State of Work, we accomplished:

1. Established multiple stable LNCaP.iAKT sublines for future experiments;
2. Established few stable LNCaP.iAKTkm sublines that expressing a mutant kinase-dead iAKT for control experiments;
3. Established few stable LNCaP.Neo sublines bearing the empty vector only for control purpose;
4. Evaluated the signaling capacity of the LNCaP.iAKT stable cell lines, including CID-mediated iAKT phosphorylation (pAkt S⁴⁷³) and activation (kinase activity towards to GSK-3 α^{21} / β^9 phosphorylation).

Reportable Outcomes

LNCaP sublines:

- 6 clones of LNCaP.iAKT cell lines;
- 2 clones of LNCaP.iAKTkm cell lines;
- 3 clones of LNCaP.Neo cell lines

Conclusion

In this first year period, we completed the proposed work according to the State of Work (month 0-12). As the first step, we established the LNCaP sublines expressing the iAKT system construct as well as the control vectors. We demonstrated that the established LNCaP.iAKT cells showed a promising ability that the iAKT can be activated after the cells are treated by the dimerization inducer AP22783, and the activated iAKT (F_{pk3}- Δ phAKT) is functionally capable to perform its physiological task, phosphorylating the downstream target GSK-3. In the next year or step, we will used those LNCaP subline cells to determine whether CID-mediated activation of iAKT promotes survival/proliferation of prostate cancer LNCaP cells after androgen withdrawal.

Reference

1. Jemal A, Thomas A, and Murray T. Cancer statistics. (2002) CA Cancer J Clin 52:23-47.
2. Huggins C and Hodges CV. (1941) Studies on prostatic cancer: The effects of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. Cancer Research 1:293-297.
3. Denmeade SR, Lin XS and Isaacs JT. (1996) Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. Prostate 28(4):251-65.
4. Graff JR, Konicek BW, McNulty AM, Wang Z, Houck K, Allen S, Paul JD, Hbailu A, Goode RG, Sandusky GE, Vessella RL and Neubauer BL. (2000) Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27Kip1 expression. J Biol Chem 275(32):24500-5.
5. Murillo H, Huang H, Schmidt LJ, Smith DI and Tindall DJ. (2001) Role of PI3K signaling in survival and progression of LNCaP prostate cancer cells to the androgen refractory state. Endocrinology 142(11):4795-805.

6. Sun M, Wang G, Paciga JE, Feldman RI, Yuan ZQ, Ma XL, Shelley SA, Jove R, Tsichlis PN, Nicosia SV and Cheng JQ. (2001) AKT1/PKB α kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. *Am J Pathol* 159(2):431-437.
7. Zinda MJ, Johnson MA, Paul JD, Horn C, Konicek BW, Lu ZH, Sandusky G, Thomas JE, Neubauer BL, Lai MT and Graff JR. (2001) AKT-1, -2, and -3 are expressed in both normal and tumor tissues of the lung, breast, prostate, and colon. *Clin Cancer Res* 7(8):2475-2479.
8. Nakatani K, Thompson DA, Barthel A, Sakaue H, Liu W, Weigel RJ and Roth RA. (1999) Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. *J Biol Chem* 274(31):21528-32.
9. Mousses S, Wagner U, Chen Y, Kim JW, Bubendorf L, Bittner M, Pretlow T, Elkahoul AG, Trepel JB and Kallioniemi OP. (2001) Failure of hormone therapy in prostate cancer involves systematic restoration of androgen responsive genes and activation of rapamycin sensitive signaling. *Oncogene* 20(46):6718-23.
10. Datta SR, Brunet A and Greenberg ME. (1999) Cellular survival: a play in three Akts. *Gens Dev* 13:2905-2927.
11. Chan TO, Rittenhouse SE and Tsichlis PN. (1999) AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu Rev Biochem* 68:965-1014.

Appendices

A previous publication describing the creation of the iAKT system is attached to this report.

1. **Benyi Li**, Desai, S.A., MacCorkle-Chosnek, R.A., Fan, L. and Spencer, D.M. A novel conditional Akt 'survival switch' reversibly protects cells from apoptosis. *Gene Ther* 2002; 9(4):233-244.

RESEARCH ARTICLE

A novel conditional Akt 'survival switch' reversibly protects cells from apoptosis

B Li, SA Desai, RA MacCorkle-Chosnek, L Fan and DM Spencer

Department of Immunology, Baylor College of Medicine, Houston, TX, USA

The anti-apoptotic Akt kinase is commonly activated by survival factors following plasma membrane relocalization attributable to the interaction of its pleckstrin homology (PH) domain with phosphatidylinositol 3-kinase (PI3K)-generated $PI3,4-P_2$ and $PI3,4,5-P_3$. Once activated, Akt can prevent or delay apoptosis by phosphorylation-dependent inhibition or activation of multiple signaling molecules involved in apoptosis, such as BAD, caspase-9, GSK3, and NF- κ B and forkhead family transcription factors. Here, we describe and characterize a novel, conditional Akt controlled by chemically induced dimerization (CID). In this approach, the Akt PH domain has been replaced with the rapamycin (and FK506)-binding domain, FKBP12, to make F3- Δ PH.Akt. To effect membrane recruitment, a myristoylated rapamycin-binding domain from FRAP/mTOR, called M-FRB, binds to lipid permeable rapamycin (and non-bioactive synthetic 'rapalogs'), leading to reversible heterodimerization of M-FRB with

FKBP- Δ PH.Akt. Like endogenous c-Akt, we show that the kinase activity of membrane-localized F3- Δ PH.Akt correlates strongly with phosphorylation at T308 and S473; however, unlike c-Akt, phosphorylation and activation of inducible Akt (iAkt) is largely PI3K independent. CID-mediated activation of iAkt results in phosphorylation of GSK3, and contributes to NF- κ B activation in vivo in a dose-sensitive manner. Finally, in Jurkat T cells stably expressing iAkt, CID-induced Akt activation rescued cells from apoptosis triggered by multiple apoptotic stimuli, including staurosporine, anti-Fas antibodies, PI3K inhibitors and the DNA damaging agent, etoposide. This novel inducible Akt should be useful for identifying new Akt substrates and for reversibly protecting tissue from apoptosis due to ischemic injury or immunological attack.

Gene Therapy (2002) 9, 233–244. DOI: 10.1038/sj/gt/3301641

Keywords: Akt; CID; apoptosis; survival switch; conditional signaling protein; inducible Akt

Introduction

When growth factors are limiting in the extracellular milieu, most cell types die by apoptosis due to finely tuned homeostatic mechanisms. One common pathway by which ligand-bound growth factor receptors prevent apoptosis is through the phosphorylation-dependent membrane recruitment and activation of phosphatidylinositol 3-kinases (PI3K). PI3Ks generate phosphatidylinositol 3,4-diphosphate ($PtdIns(3,4)P_2$) and $PtdIns(3,4,5)P_3$ by phosphorylating the D-3 position of the inositol ring of phosphoinositides. In turn, these 3-phosphorylated lipids can lead to the plasma membrane recruitment and activation of a number of cytosolic signaling molecules by binding to their pleckstrin homology (PH) domains. The importance of the PH domain is underlined by its recent discovery in over 250 genes, the 11th most common InterPro family found in the human proteome.¹ Although the cellular responses regulated by PI3Ks are diverse, including growth, survival, transformation, vesicle trafficking, and others (reviewed in Ref. 2), activation of the serine/threonine kinase Akt/PKB (the cellular homologue of the viral oncogene, v-Akt), appears to be

central to the PI3K-mediated delay of apoptosis and increase of cell survival.³

Although c-Akt was cloned a decade ago,⁴ the mechanism by which Akt propagates survival signals in eukaryotic cells has only been elucidated more recently (reviewed in Ref. 5). All three mammalian isoforms of Akt (Akt1/PKB α /RAC-PK α , Akt2/PKB β /RAC-PK β , and Akt3/PKB γ /RAC-PK γ) have an amino-terminal PH domain, a serine-threonine (S/T) kinase domain related to protein kinase A and C (PKA and PKC) family members, and a carboxy-terminal regulatory domain. Akt is activated in response to various survival stimuli, such as growth factors, cytokines and hormones, in a PI3K-dependent manner.⁶ In addition, PI3K-independent activation of Akt has also been shown after treatment with heat shock,⁷ β -adrenergic receptor activation,⁸ PKC activation,⁹ and c-AMP up-regulation.¹⁰ It is believed that Akt activation involves three steps, in which the first step is the interaction of the inhibitory PH domain with $PtdIns(3,4)P_2$ and $PtdIns(3,4,5)P_3$, leading to membrane recruitment and a conformational change in the kinase. Together these two events expose T308 (based on Akt1) in the activation loop of the catalytic domain to the constitutively active, $PtdIns(3,4,5)P_3$ -dependent kinase-1 (PDK1). Finally, T308 phosphorylation leads to phosphorylation in the regulatory domain at S473 (Akt1) by PDK2. Although PDK2 is still poorly defined, PKC members, integrin-linked kinase (ILK), PDK1 (bound to

PRK2¹¹), and Akt autophosphorylation¹² have all been reported to be the effectors of this event (reviewed in Refs 3 and 5).

Further underlying the nodal position of Akt in survival signaling are the observations that pT308 and pS473 have a relatively short half-life *in vivo*, and phosphatase inhibitors, such as calyculin A and okadaic acid, a relatively specific inhibitor of PP2A, are able to prevent Akt dephosphorylation and inactivation.¹³ Moreover, Akt family members are up-regulated in several cancers and inactivation of the PtdIns phosphatase, PTEN, is also associated with cancer and Akt activation.¹⁴⁻¹⁹

To date, Akt has been implicated in various physiological processes including cell cycle regulation, cellular metabolism and cell survival. The first identified downstream target of Akt was glycogen synthase kinase-3 (GSK-3), which is phosphorylated at serine 21 in GSK3- α and serine 9 in GSK3- β , leading to inactivation and the up-regulation of a number of substrates involved in cellular metabolism, including glycogen synthesis.²⁰ Recently, several targets of the PI3K/Akt signaling pathway have been identified that may explain the ability of this regulatory cascade to promote survival (reviewed in Ref. 5). These targets include two components of the intrinsic cell death machinery, Bad and caspase 9, transcription factors of the forkhead family (ie AFX) that can up-regulate FasL, and the kinase, IKK α , that regulates the anti-apoptotic transcription factor, NF- κ B. Additional substrates for Akt include eNOS, phosphofructokinase-2, phosphodiesterase 3 β and the reverse transcriptase subunit of telomerase. These and other as yet unidentified Akt substrates might mediate the effects of Akt on cellular survival.

In order to elucidate the function of many signaling molecules, constitutively active or 'dominant negative' mutant proteins are often overexpressed in target cells. When Akt or many other 'upstream' signaling molecules are modified to contain a membrane targeting sequence, the increased proximity to activating kinases, such as PDK1, or to membrane-localized substrates typically leads to the constitutive phenotype. For example, most functional Akt studies have utilized either Src family myristoylation-targeting peptides or the myristoylated gag sequence within v-Akt. Under these conditions, however, the kinase is activated as soon as it is expressed in cells, but the effects of activation may not be monitored until much later, when the direct effects of Akt are typically obscured.

For controlled gene expression or kinase activation, several approaches are available such as tetracycline-regulatable transcription systems,²¹ chimeras of hormone binding domains (HBD) with target proteins²²⁻²⁴ and chemically induced dimerization (CID).^{25,26} The CID system uses synthetic bivalent ligands to rapidly crosslink signaling molecules that are fused to ligand-binding domains.

CID has been used to trigger the oligomerization and activation of cell surface,^{25,27,28} or cytosolic proteins,^{29,30} the recruitment of transcription factors to DNA elements to modulate transcription,^{31,32} or the recruitment of signaling molecules to the plasma membrane to simulate signaling.^{33,34} Here, we extend these applications with the development of a highly responsive, conditional Akt molecule, called inducible Akt (iAkt) whose range of activation extends from undetectable to comparable to that of constitutively active Myr-Akt. Activation of iAkt is

based on ligand-dependent recruitment of chimeric Akt to a membrane-bound myristoylated 'docking protein'. To improve the CID-responsiveness of iAkt, we utilized novel, high specificity heterodimerizing ligands, called rapalogs, based on the immunosuppressant drug, rapamycin.³⁵ Otherwise bioinert, rapalogs can reversibly crosslink proteins fused to the proline isomerase, FKBP12, with FRB₁, a 90 amino acid rapamycin-binding domain excised from the S6 kinase kinase, FRAP/mTOR, containing L2098 to confer specificity. While rapalogs bind with high affinity to FRB₁, binding to endogenous FRAP is sterically prevented. Thus, Akt fused to FKBP_s can be efficiently recruited to the plasma membrane and activated.

Although iAkt based on full-length Akt, had very high basal activity due presumably to high CID-independent membrane association, removal of the PH domain in iAkt variant, F3- Δ PH.Akt eliminated basal activity while concomitantly permitting CID dose-dependent phosphorylation at T308 and S473 and Akt activation. Further, we show that CID-dependent Akt activation is largely PI3K independent, can greatly augment NF- κ B activity and can lead to phosphorylation of GSK3 α/β . In turn, conditional activation of iAkt leads to reversible protection from a number of apoptotic stimuli, including the PI3K inhibitors wortmannin and LY294002, the broad-spectrum protein kinase inhibitor, staurosporine, DNA damage mediated by the topoisomerase II inhibitor, etoposide, and Fas crosslinking. As gene therapy comes of age the ability to conditionally regulate viability with an anti-apoptotic 'survival switch' like iAkt is likely to be as useful as the more well-established pro-apoptotic suicide genes.

Results

CID-mediated activation of Akt/PKB

The strategy used in this study to develop conditional alleles of Akt kinase is based on the knowledge that membrane targeting of endogenous Akt family members (ie AKT1, 2, 3) leads to their phosphorylation and activation.³⁶ Similarly, several groups have shown that myristoylated (M) Akt (ie M-Akt) leads to its constitutive activation (reviewed in Ref. 5). Therefore, we used conditionally induced dimerization (CID) to reversibly recruit Akt1 to the plasma membrane. It was shown previously that rapamycin could crosslink endogenous FKBP12 with a 90 amino acid domain from FRAP/mTOR, called FRB (FRAP rapamycin binding domain, residues 2025-2113).^{37,38} More recently, 'rapalogs' have been developed that are modified to greatly reduce binding, and therefore bioactivity, to endogenous FRAP, but are able to bind with high affinity to modified FRB₁ (T2098L).³⁵ As shown in Figure 1a, the heterodimeric rapalog/CID_{HED} can effect the crosslinking of FRB₁ and FKBP12 (called F). In these experiments, we used the non-toxic variant of FKBP12, F_{pk} (FKBP12(G89P,190K)), to eliminate background toxicity.³⁰ For the plasma membrane docking protein, we fused a c-Src myristoylation signal to one or two copies of FRB₁, to create M-FRB₁ and M-FRB₁2, respectively (Figure 1b). To create inducible Akt (iAkt) molecules, three tandem FKBP domains (F3) were fused to the N- or C-termini of wild-type Akt or a variant (Δ PH.Akt), lacking the pleckstrin homology (PH)

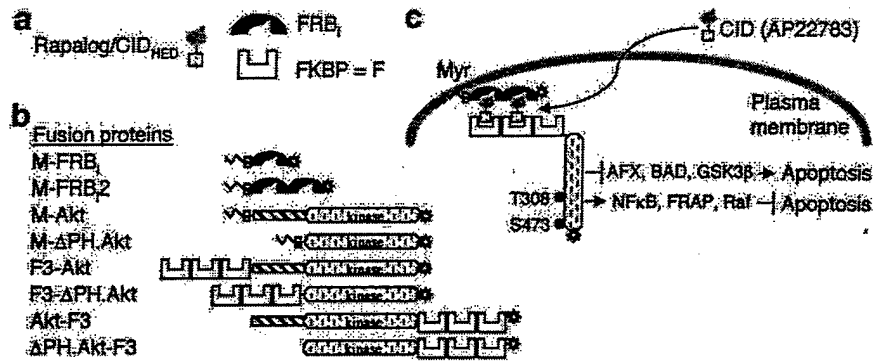


Figure 1 Schematic representation of constructs used in this study. (a) Heterodimeric (HED) CIDs/rapalogs and CID_{HED}-binding domains. Synthetic ligand, AP22783, is a bivalent compound that binds to the FRB₁ domain (but not endogenous FRAP/mTOR) and concomitantly to FKBP12 (F) variants. (b) The CID-binding domains were subcloned as monomers (FRB₁), dimers (FRB₁₂), or tandem trimers (F3) into expression vectors to generate chimeric proteins. The c-Src myristoylation (M) signal sequence (horizontal bars) was fused to the N-terminus of FRB or Akt kinase alleles (vertical dashes). Wild-type or PH domain (striped) deletion mutants of Akt were fused to F3 at their N- or C-terminal ends. All constructs used in this study have an HA epitope tag (star) at their C-terminus. (c) Model of CID_{HED}-mediated membrane targeting and activation of inducible Akt (iAkt) kinase. Cell permeable rapalogs (eg AP22783) bring F3ΔPH.Akt to the plasma membrane by crosslinking FRB₁ and FKBP domains, triggering Akt phosphorylation at two key residues for activation, T308 and S473. Phosphorylation of multiple downstream substrates can rescue cells from death by inhibition of promoters of apoptosis or activation of inhibitors of apoptosis.

domain to reduce natural membrane association. Furthermore, we developed constitutively active, myristoylated Akt (M-Akt) or M-ΔPH.Akt and kinase-dead mutant versions (ie Akt.K179M, named AktKM) of chimeric Akt constructs. For identification and purification, all chimeric constructs were HA epitope-tagged (name left off for simplicity). Thus, membrane recruitment of F3-modified Akt by rapalogs, such as AP22783 used in these experiments, leads to phosphorylation of Akt at T308 and S473, induction of Akt kinase activity, and phosphorylation of downstream effector molecules, leading to modulation of apoptotic signals.

Membrane targeting of PH domain-less Akt leads to rapalog dose-dependent activation of NF-κB

Two key requirements for efficient synthetic regulation of a biological event are highly specific conditional dependency and low background. NF-κB induction is a major target of Akt following growth factor signaling, and multiple reports show that a constitutively active myristoylated Akt (M-Akt) can enhance protein kinase C (PKC)-mediated NF-κB induction by either phosphorylation of IKKα,³⁹ the activation domain of p65/RelA,⁴⁰ or both.⁴¹ Therefore, in order to optimize iAkt, we hypothesized that the ability of iAkt variants to complement PKC-induced NF-κB transcriptional activity using an NF-κB-responsive secreted alkaline phosphatase (SEAP) reporter plasmid would be a sensitive assay for Akt activation.

To validate this assay, the human T cell line, Jurkat-TAg, was cotransfected with reporter plasmid, NF-κB/SEAP, along with constitutively active M-Akt expression vector or empty control vector. Twenty-four hours after transfection, cells were divided into aliquots that were stimulated with sub-optimal levels (5 ng/ml) of the phorbol ester, PMA, or were untreated. After an additional 24 h, SEAP activity was measured. Although Akt activity alone was insufficient to induce measurable NF-κB activity, M-Akt expression could greatly potentiate (by three- to four-fold) PKC-induced NF-κB activity, consistent with multiple reports. Furthermore, inhibition of PI3K by LY294002 (5 μM) or wortmannin (1 μM) did not prevent NF-κB activation by M-Akt plus PKC,

although inhibition of 'typical' PKC isoforms with R0318220 (1 μM) led to complete inhibition of NF-κB as expected (Figure 2).

Since the constitutively active Akt (T308) kinase, PDK1, is primarily membrane-associated following growth factor stimulation, membrane recruitment of Akt via its PH domain is necessary for its activation.^{42–44} Furthermore, although the PH domain has been shown to suppress basal phosphorylation of T308 and Akt activation when not bound by its lipid ligand, PIP₂, this initial phosphorylation should still require interaction with membrane-localized PDK1.⁴⁵ Therefore, we reasoned that removing the PH domain in iAkt should theoretically lead to low basal activity and a large activation index. To test this hypothesis, we compared basal Akt activity and activation following membrane recruitment of full-length

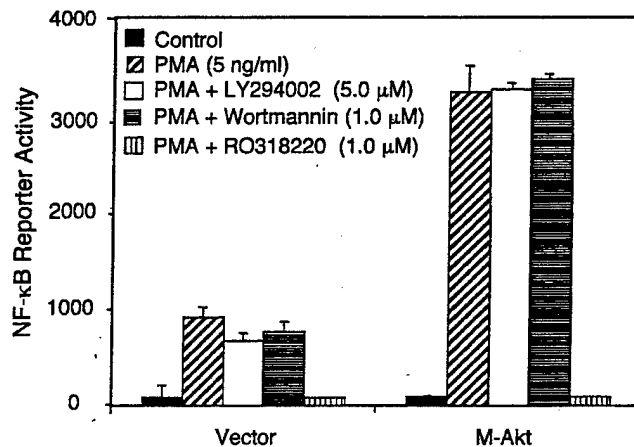


Figure 2 M-Akt enhances NF-κB induction induced by PMA. Jurkat-TAg cells were cotransfected with NF-κB/SEAP reporter plasmid along with control vector or M-Akt expression vector by electroporation. After 24 h, cells were treated with sub-maximal levels (5 ng/ml) of PMA plus PI3K inhibitor, LY294002 (5 μM) or wortmannin (1 μM), PKC inhibitor, R0318220, (1 μM), or control diluent. SEAP activity was measured 24 h later as described in Materials and methods. Data shown are the mean and s.d. of two duplicate experiments.

and truncated Akt, lacking the PH domain, in our NF- κ B reporter assay.

We fused both full-length and Δ PH.Akt to a tandem trimer of the CID-binding domain, F3, at both the amino and carboxyl termini. As before, Jurkat-TAg cells were cotransfected with reporter plasmid NF- κ B/SEAP along with the membrane docking molecule, M-FRB₂, alone, various F3-Akt chimeras, alone, or both together. Twenty-four hours after transfection, cells were stimulated with 5 ng/ml PMA along with log dilutions of heterodimerizing CID, AP22783. After additional 24 h incubation, SEAP activity was assayed. As shown in Figure 3a, wild-type Akt showed significant CID-independent NF- κ B induction that was only slightly increased by crosslinking to the membrane, via M-FRB₂. This was true regardless of whether F3 was fused to the N- or C-terminus (not shown) of Akt. As expected, membrane recruitment or overexpression of kinase-deficient Akt.KM (K179M) had no detectable effect on NF- κ B induction over PMA alone. Thus, membrane recruitment of full-length Akt only slightly increases its activity due to the high basal activity from its overexpression.

In contrast, membrane recruitment of F3- Δ PH.Akt showed a very clear CID-dependent induction of NF- κ B with undetectable CID-independent activity (Figure 3b). Moreover, myristoylated M- Δ PH.Akt was more active than M-Akt in augmenting NF- κ B activation, consistent with an inhibitory function for the PH domain. Again, M-FRB₂ alone or recruitment of kinase dead F3- Δ PH.Akt.KM did not influence NF- κ B induction (Figure 3b and not shown). These results indicate that the chimeric F3- Δ PH.Akt allele is strongly CID-inducible with very low basal activity. Also, these results are consistent with previous reports that the PH domain of Akt kinase is responsible for its translocation to the plasma membrane and also has an inhibitory function.

Since most applications of CID technology have been based partly, at least, on empirically designed inducible chimeric proteins, CID-mediated targeting or crosslinking might not always faithfully reflect physiological sig-

naling. Further, CID-binding domains, like FKBP12, could potentially sterically hinder an essential target protein domain(s). Therefore, we tested i Δ PH.Akt with F3 fused to both termini of Δ PH.Akt. As shown in Figure 4a, the N-terminal fusion chimera, F3- Δ PH.Akt, potentiated NF- κ B transactivation somewhat better than the C-terminal chimera, Δ PH.Akt-F3. Since both molecules were expressed at similar levels (not shown), membrane recruitment of F3- Δ PH.Akt may place Akt in a more favorable orientation for interacting with PDK1 or other interacting proteins. In either orientation, however, both iAkt versions were devoid of detectable basal NF- κ B signaling.

Since M-FRB₂ could potentially recruit two chimeric Akt molecules simultaneously, we wanted to determine if membrane recruitment of one Akt molecule was sufficient for optimal activation or whether oligomerization of multiple Akt molecules might enhance activation. Therefore, we compared CID-mediated iAkt activity when the membrane docking molecule, contained one or two FRB₁ domains (FRB₁ versus FRB₂, respectively). As shown in Figure 4b, there was no significant difference in NF- κ B induction by iAkt whether one or two tandem FRB₁ domains were used for the docking site, indicating that forced Akt oligomerization is not a prerequisite for its activation. Nevertheless, this experiment does not rule out that Akt crosslinking might contribute to its activation under normal circumstances.

CID-dependent membrane targeting of Akt kinase results in rapid phosphorylation and activation

Following membrane targeting of endogenous Akt-1, phosphorylation at two highly conserved residues, T308 in the activation loop, and S473 in the inhibitory domain, occurs by PDK1 and PDK2, respectively.⁴⁶ To directly address whether CID-mediated membrane targeting of iAkt also leads to phosphorylation at those sites, 293T cells were transiently co-transfected with M-FRB₂ and F3- Δ PH.Akt expression plasmids. After 24 h, transfected cells were serum starved for another 24 h and then

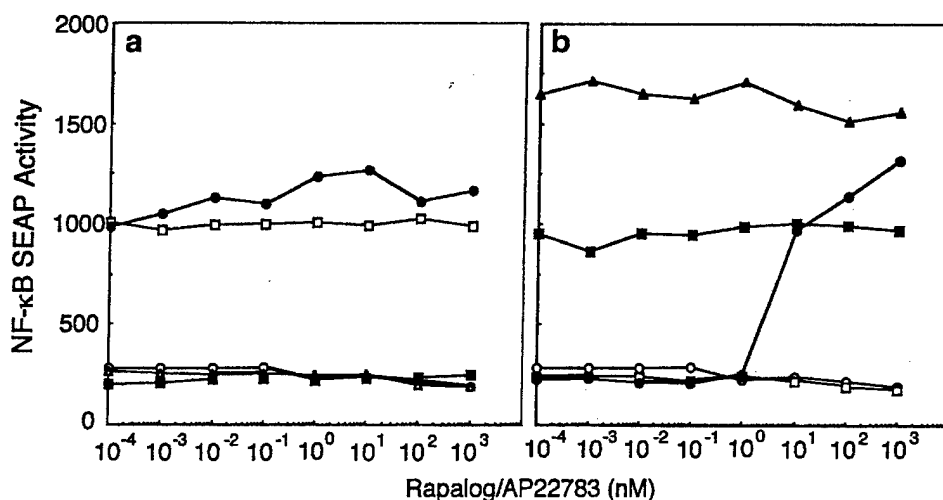


Figure 3 CID-mediated membrane targeting of Δ PH.Akt, but not wild-type Akt, induces titratable NF- κ B transactivation. (a and b), Jurkat-TAg cells were transiently cotransfected with reporter plasmid NF- κ B/SEAP along with (a) M-FRB₂ (open circle), F3-Akt (open square), F3-Akt.KM (triangle), M-FRB₂ + F3-Akt (closed circle), M-FRB₂ + F3-Akt.KM (closed square), or (b) M-FRB₂ (open circle), F3- Δ PH.Akt (open square), M-FRB₂ + F3- Δ PH.Akt (closed circle), M-Akt (closed square), or M- Δ PH.Akt (triangle). After 24 h, cell aliquots were treated with PMA (5 ng/ml) plus half-log dilutions of the rapalog, AP22783. SEAP activity was measured 24 h later and reported directly. Data are representative of at least two experiments.

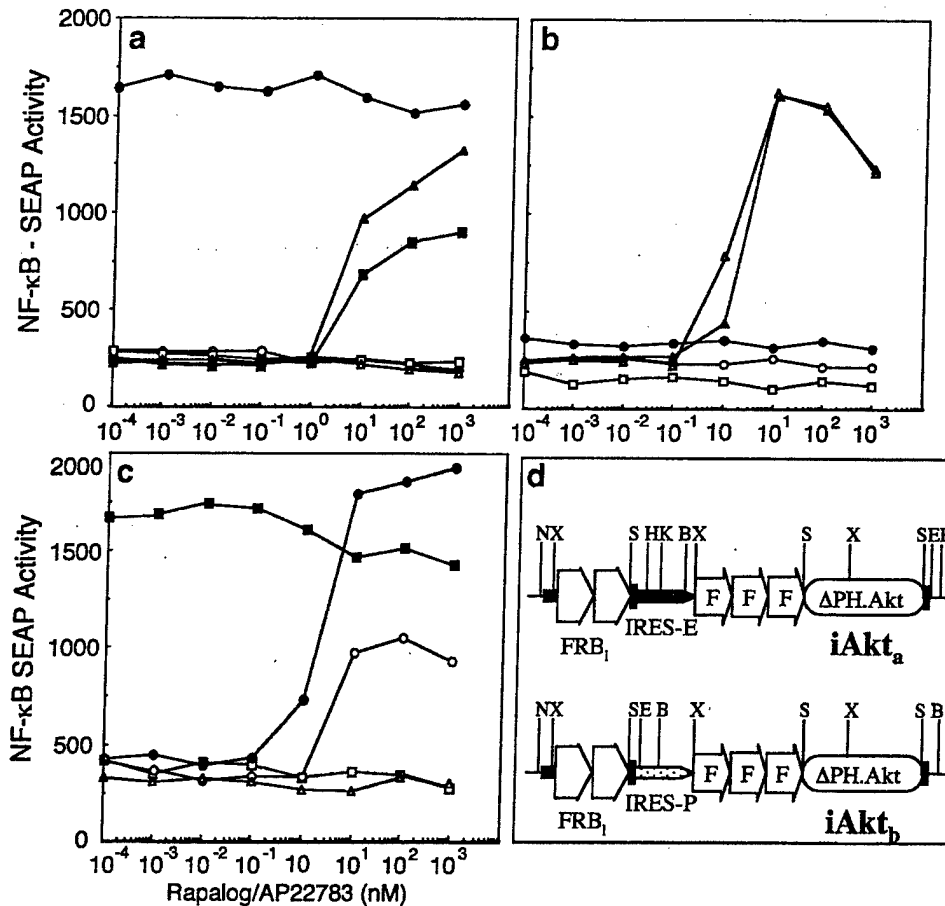


Figure 4 Optimization of iAkt based on CID-mediated NFκB induction. (a–c) Jurkat-TAg cells were transiently cotransfected with NFκB/SEAP along with (a) M-FRB₂ (open circle), ΔPH.Akt-F3 (open square), F3-ΔPH.Akt (open triangle), M-FRB₂ + ΔPH.Akt-F3 (closed square), M-FRB₂ + F3-ΔPH.Akt (closed triangle), M-ΔPH.Akt (closed circle), or (b) F3-ΔPH.Akt (closed circle), M-FRB₂ (open circle), M-FRB₂ + F3-ΔPH.Akt (closed triangle), M-FRB₂ + F3-ΔPH.Akt (open triangle), or (c) M-FRB₂ (triangle), F3-ΔPH.Akt (open square), iAkt_a (open circle), iAkt_b (closed circle), or M-ΔPH.Akt (closed square), and treated as above. (a) The orientation of Akt and FKBP has only a minor influence on efficacy. (b) Membrane recruitment of iAkt without oligomerization is sufficient for activation. (c) Bicistronic vector, iAkt_b, containing M-FRB₂ and F3-ΔPH.Akt separated by the poliovirus IRES, functions more efficiently than iAkt_a, that uses the EMCV IRES. For each experiment, data are representative of three individual experiments. (d) Schematic (scaled) of iAkt and iAkt_b. M-FRB₂-E and F3-ΔPH.Akt are separated by EMCV (E) or polio (P) IRES sequences, respectively, in expression vector pSH1 (not shown). Myristoylation (M), horizontal black bar. HA epitope (E), vertical black bar. N, NcoI; X, XhoI; S, SalI; H, HindIII; K, KpnI; B, BamHI; E, EcoRI.

treated with AP22783. As shown in Figure 5a, AP22783 treatment greatly stimulated interaction with phospho-specific antibodies against Akt S473 and T308 sites as early as 30 min after drug addition. Although 120 min serum (10% FBS) treatment stimulated phosphorylation of the endogenous protein, serum did not stimulate phosphorylation of iAkt during this period. Curiously, activation of iAkt led to increased phosphorylation of endogenous Akt, particularly at S473. This is not likely to be an effect of AP22783, since this does not happen in control-transfected cells. Therefore, it is likely that activation of iAkt can lead to partial activation of endogenous Akt. These results demonstrate that CID-mediated membrane targeting of iAkt chimeras stimulates Akt phosphorylation.

For targeting tissues or cell lines with bigenic inducible proteins, multi-cistronic vectors can be used to ensure that two, or more, proteins are coexpressed; however, the efficiency of distinct internal ribosome entry sequences (IRES) can be tissue-specific. Therefore, we developed two different bicistronic iAkt vectors, using either the

commonly used EMCV IRES, called iAkt_a, or iAkt_b, using the less characterized IRES from poliovirus. Following electroporation of bicistronic vectors into Jurkat cells and AP22783 stimulation, we observed consistently higher NF-κB induction by iAkt_b compared with iAkt_a (Figure 4c). Therefore, iAkt_b was used to create variant Jurkat lines stably expressing both FRB₂ and F3-ΔPH.Akt, called Jurkat.iAkt cells.

To further measure the induction of Akt enzymatic activity, Jurkat.iAkt cells were serum starved for 24 h and thereafter treated with AP22783 or serum for 30 min. Chimeric F3-ΔPH.Akt kinase was immunoprecipitated with the anti-HA antibody and Akt kinase activity was measured using an *in vitro* kinase assay that uses a GSK3α/β 'crosstide' as a substrate. The level of phosphorylated GSK3 crosstides was determined by immunoblotting with phospho-specific antibody against GSK3-α21/β9. As shown in Figure 5b, only AP22783 treatment, but not serum or mock treatment, was associated with GSK3 phosphorylation in this assay, indicating, as above, Akt activation after CID-mediated membrane targeting.

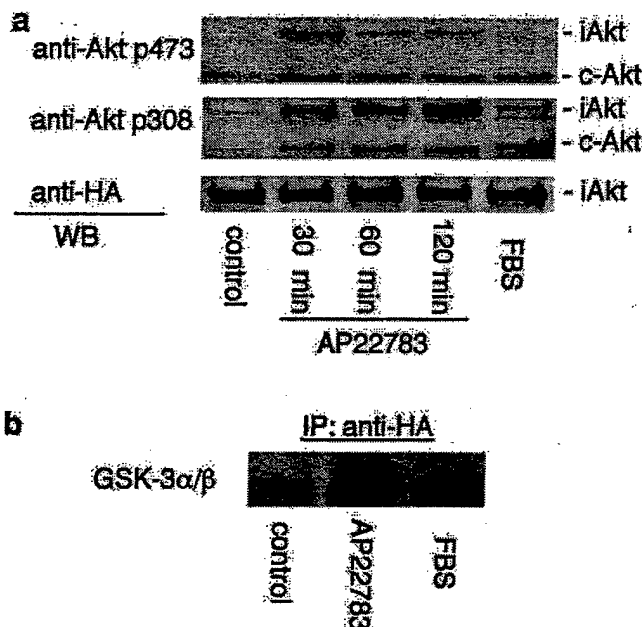


Figure 5 Phosphorylation and activation of iAkt following CID-mediated membrane targeting. (a) 293T cells were cotransfected with M-FRB₂ plus F3-ΔPH.Akt followed by 24 h of serum starvation. Thereafter, cells were untreated (negative control), treated with FBS for 120 min (positive control) or AP22783 for 30, 60 or 120 min. Western blot using anti-phospho-specific antibodies against Akt T308 or S473 measured the extent of phosphorylation of endogenous Akt or iAkt. Anti-HA blotting served as a loading control. c-Akt, endogenous Akt. (b) Jurkat.iAkt cells were serum-starved for 24 h followed by treatment with AP22783 or serum for 30 min. iAkt was immunoprecipitated from cell lysates using anti-HA antibody and incubated with the GSK-3 'cross-tide' as a substrate. Phosphorylated GSK3 cross-tides were quantified by SDS-PAGE separation and immunoblotting with a phospho-specific antibody against GSK-3α21/β9.

CID-mediated membrane targeting of iAkt results in PI3K-independent activation

For maximum utility, an ideal CID-inducible protein would respond only to CID, but not to environmental signals, such as growth factors. Since endogenous Akt is a major effector molecule of PI3K signaling,⁶ inhibition of PI3K leads to inhibition of c-Akt. However, the activity of membrane targeted Akt, such as M-Akt, is largely PI3K independent, presumably because basal levels of membrane-associated PDK1 are sufficient for Akt phosphorylation. To determine if CID-mediated Akt activation is also largely PI3K independent, the effects of PI3K inhibition, using two different inhibitors, were studied in the NF-κB reporter assay described above. Jurkat-TAg cells were cotransfected with reporter plasmid, NF-κB/SEAP, along with the bicistronic plasmid iAkt_t. After 24 h, cells were pretreated with two different concentrations of either wortmannin (1 μM and 10 μM) or LY294002 (5 μM and 50 μM) for 40 min, and then cell aliquots were stimulated with 5 ng/ml PMA and log dilutions of AP22783. SEAP activity was measured 24 h later. As shown in Figure 6a and b, the inhibitors at either concentration did not prevent NF-κB induction by iAkt, although maximal NF-κB activation was moderately effected.

To further compare inhibition of endogenous Akt with iAkt, Jurkat.iAkt cells were serum starved for 24 h followed by 30 min pretreatment with PI3K inhibitors, wortmannin and LY294002, or MEK inhibitor, PD98059, as a control. After inhibitor pretreatment, AP22783 was added

to the media for another 30 min to mobilize membrane recruitment of iAkt. As shown in Figure 6c, addition of either PI3K inhibitor significantly blocked endogenous Akt phosphorylation at T308, but had a much smaller, if any (for 1 μM wortmannin), effect on iAkt phosphorylation. The MAPK signaling inhibitor, PD98059, had no discernable effect on either endogenous or iAkt. These results, together with the NF-κB/SEAP assay (Figure 6a, b), indicate that CID-mediated iAkt activation is primarily independent of environmental signaling.

CID-mediated activation of iAkt leads to apoptosis resistance following multiple pro-apoptotic signals

When overexpressed, wild-type or constitutively active Akt has been demonstrated to protect cells from a variety of apoptotic stimuli, including treatment with DNA-damaging agents, PI3K inhibitors, Fas-crosslinking, UV (or γ) irradiation, c-myc overexpression, growth factor withdrawal, TGFβ treatment, matrix detachment, or cell cycle perturbation (reviewed in Ref. 5). To test whether CID-mediated activation of chimeric Akt could also protect cells from apoptosis, Jurkat.iAkt cells were given various apoptotic insults in the presence or absence of rapalogs.

Initially, we assessed the protective effects of iAkt on staurosporine (STS)-induced apoptosis. Although staurosporine is a potent inhibitor (IC₅₀ ~3 nM) of many PKC family members,⁴⁷ it can also inhibit other S/T and tyrosine kinases at higher concentration.⁴⁸ Therefore, the exact mechanism of triggering apoptosis is likely to be complex. Following serum starvation for 24 h, Jurkat.iAkt cells were treated with 0, 0.5 or 2.0 μM STS with or without AP22783 (400 nM) for 6 h in the absence of serum. Cell death was monitored using propidium iodide (PI) staining and flow cytometry (FCM) by quantitation of subdiploid cells. As expected, STS induced cell death in a dose-response manner (Figure 7a). At low-dose STS treatment, 19.4% of cells were apoptotic after 6 h, but this toxicity could be fully blocked by AP22783 treatment. Further, although high-dose STS treatment triggered greater apoptosis, iAkt activation was able to prevent, or delay, apoptosis for the majority (36% to 17% without background correction) of 2 μM STS-treated cells.

To further confirm the anti-apoptotic effect of iAkt activation on STS-induced cell death, caspase-3 activation and PARP substrate cleavage were measured by immunoblotting. As shown in Figure 7b, STS treatment at low- and high-dose resulted in dramatic PARP cleavage and significant reduction of procaspase-3 (evidence for procaspase-3 activation) at high-dose. However, AP22783 addition blocked STS-induced caspase-3 activation and low-dose STS-induced PARP cleavage, and also reduced high-dose STS-induced PARP cleavage. Thus, activation of iAkt can block or, at minimum, delay apoptosis by the potent apoptosis inducer, STS.

As shown in Figure 6, CID-mediated activation of chimeric ΔPH.Akt and induction of NF-κB are largely independent of PI3K. To further characterize the ability of iAkt activation to block apoptosis, we directly examined the effects of AP22783 treatment following PI3K inhibition of Jurkat.iAkt cells. Serum-starved Jurkat.iAkt cells were treated with half-log dilutions of wortmannin (0.03–10 μM) or LY294002 (0.3–100 μM) with or without AP22783 (400 nM) in low (2%) or high (10%) FBS-containing medium for 9 h (Figure 8a and b and not shown). In the presence of either low or high levels of FBS, the PI3K

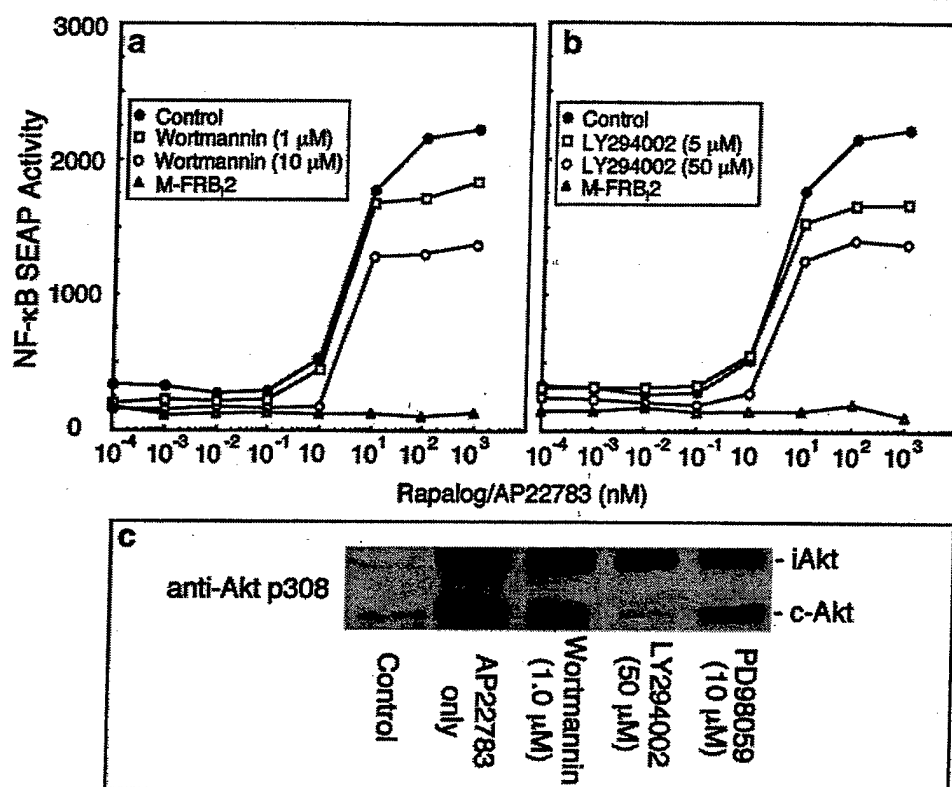


Figure 6 NF-κB transactivation induced by CID-mediated iAkt activation is PI3K independent. (a and b) Jurkat-TAg cells were transfected with bicistronic construct iAkt, and the NF-κB/SEAP reporter. Cells transfected with M-FRB2 served as a negative control. After 24 h, cells were treated with half-log dilutions of AP22783 in PMA (5 ng/ml)-containing media plus or minus PI3K inhibitors, wortmannin (a) or LY294002 (b) at two concentrations. SEAP activity was measured 24 h later, and data are representative of two experiments. (c) PI3K inhibitors prevent activation of c-Akt, but not iAkt. Following serum starvation for 24 h, Jurkat.iAkt cells were pretreated for 30 min with PI3K inhibitors, wortmannin (1 μM) or LY294002 (50 μM), or the MEK inhibitor PD98059 (10 μM) followed by incubation with AP22783 (400 nM) for an additional 30 min. Akt phosphorylation was determined by immunoblotting with phospho-specific antibody against Akt/T308.

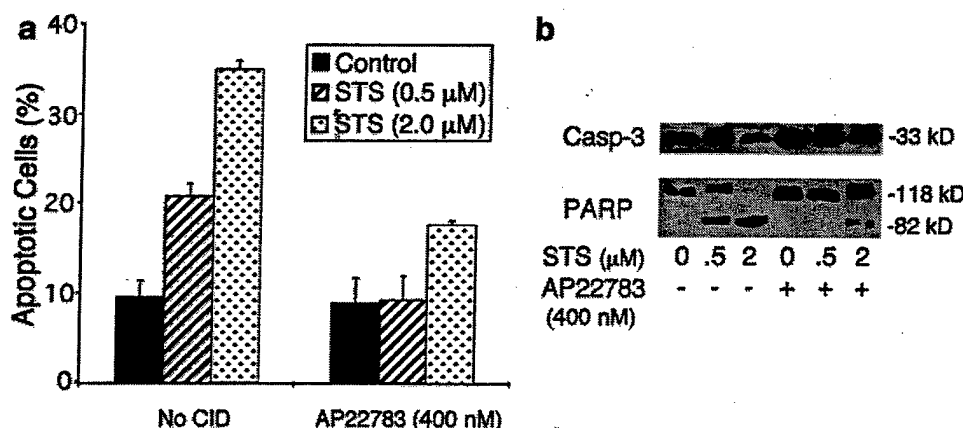


Figure 7 CID-mediated activation of Akt kinase blocks staurosporine (STS)-induced caspase-3 activation, PARP cleavage, and apoptosis. (a) Following serum starvation for 24 h, Jurkat.iAkt cells were treated with STS (0.5 or 2.0 μM) with or without (control) AP22783 (400 nM) for 6 h in serum-free conditions. Hypodiploid/apoptotic cells were determined by flow cytometry after PI staining of permeabilized cells. (b) Following serum starvation for 24 h, Jurkat.iAkt cells were treated with different doses of STS (0.5 and 2.0 μM) with or without AP22783 for 6 h in serum-free condition. Caspase-3 activation was determined by Western blotting using anti-caspase-3 antibodies as a decrease in inactive pro-caspase-3, and PARP (full-length and processed) was detected using anti-PARP antibodies.

inhibitors induced increasing cell death in a very clear dose-dependent manner. In the presence of AP22783, however, cell death was almost totally blocked regardless of the STS concentration. These results further demonstrate that CID-mediated Akt activation can mitigate PI3K inhibition-induced cell death.

It is well established that oligomerization of the Fas receptor can result in rapid formation of the cytoplasmic death-inducing signaling complex (DISC) and activation of a caspase cascade that leads to apoptosis. Recent data have revealed that activation of the PI3K-Akt pathway can protect cells from Fas-mediated death.^{49,50} Therefore,

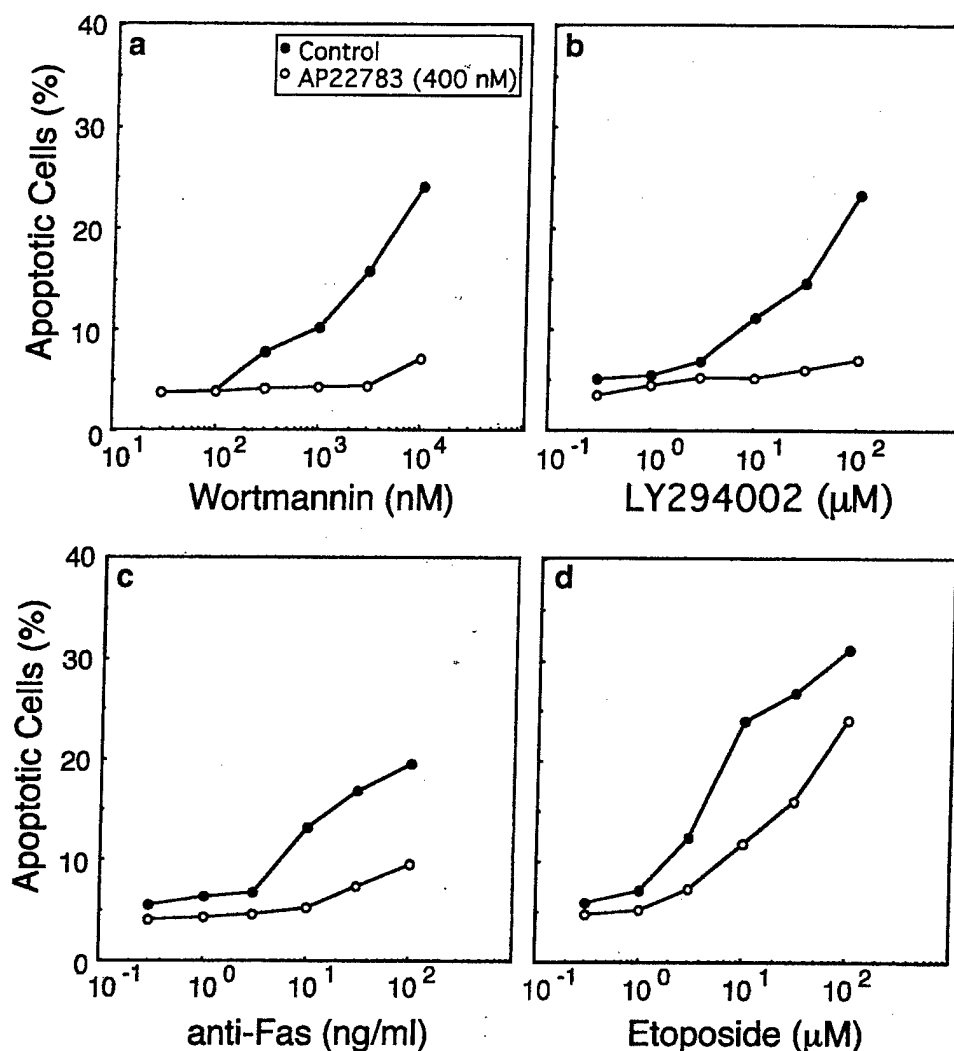


Figure 8 CID-mediated activation of Akt kinase blocks apoptosis triggered by multiple stimuli. Following serum starvation for 24 h, Jurkat.iAkt cells were treated with half-log dilutions of (a) wortmannin (0.03–10 μ M), (b) LY294002 (0.3–100 μ M), (c) anti-Fas antibody, CH-11 (0.3–100 ng/ml), or (d) etoposide (0.3–100 μ M) with or without AP22783 (400 nM) for 6 (c, d) or 9 (a, b) h in 10% FCS media. Apoptotic cells were measured by flow cytometry for subdiploid populations after PI staining.

to further investigate the potential utility of iAkt activation, we tested the effect of CID-mediated Akt activation on Fas-induced apoptosis in Jurkat.iAkt cells. After 24 h serum starvation, cells were treated with half-log dilutions (0.3–100 ng/ml) of the anti-Fas antibody, CH-11, in the presence or absence of AP22783 (400 nM) in low (2%) or high (10%) FBS for 6 h, as above. As shown in Figure 8c, Fas receptor engagement induced by CH-11 resulted in apoptosis in a dose-dependent fashion in both low (not shown) and high FBS levels. However, regardless of the FBS concentration, AP22783 treatment rescued cells from CH-11-triggered apoptosis, indicating that the synthetic activation of iAkt is also able to partially protect cells from the deleterious effects of Fas signaling.

Finally, we examined the anti-apoptotic effects of iAkt on protection from direct DNA damaging agents. The antitumor agent, etoposide (VP16), is widely used as a chemotherapeutic drug that works by inhibiting DNA topoisomerase II, which can induce apoptosis in a variety of cell types (reviewed in Ref. 51). To test if etoposide-induced cell death can be blocked by CID-mediated activation of Akt kinase, Jurkat.iAkt cells were serum-

starved for 24 h followed by treatment with half-log dilutions of etoposide (0.3–100 μ M) with or without AP22783 (400 nM) for 12 h. Again, the experiments were carried out in two different culture conditions with low (not shown) or high FBS levels. As shown in Figure 8d, AP22783 addition reduced etoposide-induced cell death efficiently in high FBS conditions. A lesser, but reproducible level of protection was seen under low FBS conditions (not shown). Although the ability of iAkt to delay or block apoptosis following etoposide treatment was not as extensive as blocking apoptosis triggered by STS, PI3K inhibition or Fas ligation, these results demonstrate that the CID-activated Akt can be a powerful 'live switch' to prevent, or delay apoptosis by multiple stimuli.

Discussion

In mammals, multiple signaling molecules are focused on triggering or inhibiting apoptosis, underscoring the importance of rapid altruistic cellular suicide after obsolescence, extensive DNA damage, viral infection, or other stresses. For example, recent reports from the genome

project suggest that in humans there are at least 14 caspases, 11 Bcl-2 family members, 30 death domain-, seven death effector domain-, 20 CARD domain-, and eight BIR domain-containing proteins. Following activation by extracellular growth/survival factors, Akt appears to be a nodal upstream signaling molecule involved in inhibiting, or at least delaying apoptosis triggered by diverse stimuli. With this in mind, we developed a conditional allele of Akt, called iAkt, which can be activated following administration of a small, lipid-permeable ligand related to rapamycin.

The development of iAkt takes advantage of the mounting evidence that induced proximity is a fundamental feature of cell signaling. In order to increase the efficiency of an enzymatic reaction, enzymes, such as kinases and phosphatases are localized to regions of the plasma membrane (and/or internal membranes) along with their substrates. These interactions are typically organized by adaptor/scaffolding molecules or by membrane-targeting domains, such as the PH domain (reviewed in Refs 52 and 53). Unlike myristoylation (Myr) or prenylation-targeting CAAX domains (found on many G proteins), PH domains lead to primarily regulated membrane localization based on the PI3K-mediated accumulation of D3-phosphorylated PtdIns compounds. Using CID-mediated membrane targeting, however, all three domains can be replaced with CID-binding domains for artificial regulation. This approach to conditional regulation of cytosolic signaling proteins was first demonstrated for Src family kinases, and G-proteins using the nonspecific homodimeric CID, FK1012, that binds equally well to endogenous and ectopically introduced FKBP12 domains.²⁵ In contrast, rapalogs bind specifically to FRB₁, permitting more exact control of heterodimerization. Furthermore, the compact size of rapalogs has desirable consequences with regards to solubility, biodistribution and membrane permeability.

Since the primary Akt kinase, PDK1, is also recruited to the plasma membrane via its PH domain, it appears somewhat unexpected that CID-activated iAkt, or even Myr-Akt alleles, should be insensitive to PI3K activation. Nevertheless, most reports suggest that Myr-Akt is largely PI3K independent. One likely explanation is that the basal level of membrane-associated PDK1 is low, but high enough for stochastic interactions with membrane-localized Akt and phosphorylation of T308 to accumulate. Once activated, Akt is ultimately down-regulated due to normal homeostatic feedback mechanisms that include reducing D-3-phosphorylated PtdIns.¹⁷ This mode of regulation should be circumvented by the removal of the Akt PH domain along with a second function, inhibition of access to T308 (reviewed by Ref. 5). The PH domain has also been associated with multimerization of Akt, which may be important for auto-phosphorylation at S473.⁵⁴ However, membrane localization, even in the absence of direct crosslinking, may evoke a threshold level of stochastic Akt interactions for the accumulation of S473 phosphorylation and hence full activation.

The other fortuitous surprise was the extremely low background of iAkt. Initial attempts by ourselves and others to design a conditional Akt using CID technology failed, probably due to the high basal activity of overexpressed full-length Akt (Figure 3a) or the inefficiency of rapamycin-based heterodimerization combined with any

biological activities of rapamycin at the doses used.⁵⁵ Interestingly, Roth and colleagues were able to design a 4-hydroxytamoxifen-inducible Akt by fusing a constitutively active Myr-ΔPH.Akt to a mutant hormone-binding domain from the estrogen receptor.⁵⁵ The availability of two distinct approaches to make conditional signaling molecules should permit independent activation of Akt and other signaling molecules in the same cell.

Although a number of applications of CID technology have been reported using high-specificity homodimerizing CIDs (CID_{hcd}), or heterodimerization with bioactive rapamycin, this study represents the first demonstration of regulating a cytoplasmic signaling molecule with bioinert CID_{hed} first described for use in activating transcription.⁵⁶ This new application should also be useful for activating multiple signaling molecules that are normally regulated by membrane localization. This includes not only proteins with membrane targeting, PH, myristoylation, or prenylation (eg CAAX) domains, but also proteins that are recruited to other membrane-localized molecules via protein-protein interaction domains, such as SH2, PTB, or SH3 domains. For example, we have also made highly inducible Src family kinases (eg Fyn, Lck) by replacing their myristoylation-targeting domains with FKBP12 and recruiting these chimeric proteins to M-FRB₁2 using rapalogs (unpublished results).

The development of iAkt and other inducible signaling molecules should have broad utility. The low basal activity and rapid induction of Akt should permit genome-wide identification of Akt substrates that might be otherwise down-regulated, dephosphorylated, or indirectly phosphorylated in cells carrying constitutively active Akt alleles. Further, since Akt is often up-regulated in various tumors, including breast, ovarian and prostate, and v-Akt is an oncogene, transgenic models bearing tissue-specific inducible Akt should lead to multiple neoplastic models.⁵⁷ Further, regulating the survival of gene-modified cells following transplantation with an Akt-based 'survival switch' may complement the use of suicide genes,^{58,59} permitting an additional dimension of control for gene therapy.

Considerable attention has focused recently on protection of multiple (eg brain, liver, heart) tissues from damage that follows ischemia and reperfusion. Many of these efforts are focused on the use of viral vectors to transfer anti-apoptotic genes. While overexpression of Bcl-2 has been the most commonly used gene for this purpose,⁶⁰⁻⁶⁴ constitutively active Myr-Akt has also been employed to protect animals from injury, such as transient cardiac ischemia.⁶⁵ Similarly, hyperoxia-induced apoptosis can also be reduced using Myr-Akt.⁶⁶ Further, several groups have shown that tissue grafts or gene-modified target tissue may better be able to withstand cellular and humoral immunity following transfer of Bcl-2.⁶⁷⁻⁷⁰ While these previous studies clearly demonstrate the potential utility of preventing apoptosis during transient injury or immunogenic conditions, longer longitudinal studies are likely to temper the long-term safety prospects of overexpressing constitutively active oncogenes *in vivo*. A reversible 'anti-death switch', such as iAkt, should prove to be a safer alternative.

Materials and methods

Plasmids construction

To generate F3-Akt, F3- Δ PH.Akt, F3-AktKM and variants, Akt and Δ PH.Akt were *PfuI* (Stratagene, La Jolla, CA, USA)-amplified from pCMV6-HA-Akt⁷¹ or pCMV6-HA-Akt K179M⁷² using *Sall*-linked 5' primers, mAkt5SPH (full-length): 5'-aga ggcac aac gac gta gcc att gtg aag gag-3' or mAkt5S (truncated Δ PH): 5'-aga gtcgac acc gcc att cag act gtg gcc-3' and 3' primer, mAkt3S: 5'-aga gtcgac ggc tgt gcc act ggc tga gta g-3'. PCR products were subcloned into pCR-Blunt (Invitrogen) or pKSII⁺ (Stratagene) and sequence verified, to create pSH5/mAkt, pSH5/m Δ PH.Akt, and pKS/mAkt.KM. The 1440-bp full-length Akt and 1130-bp Δ PH.Akt fragments were removed with *Sall* and subcloned into *XhoI/Sall*-digested M-F_{pk} 3-E, or *XhoI* or *Sall*-digested S-F_{pk} 3-E, described previously,³⁰ to create M-Akt (and Akt variants), Akt-F3 (and variants) and F3-Akt (and variants). Note, all chimeric proteins contain the HA epitope (E), but the 'E' is left off (along with 'pk' subscripts) for simplicity. To generate myristoylated rapalogue-binding domains, the rapamycin binding domain (FRB) from human FRAP (res. 2025-2113; T2098L) was *PfuI*-amplified from FRAP*-AD³⁵ using primers, 5FRBX: 5'-cgat ctgcag gagatgtggcatgaaggcctgg-3' and 3FRBS: 5'-cgat gtcgac ctttgagattcgtcggaacacatg-3' and subcloned into pCR-Blunt to produce pSH5/FRB₁. One or two copies of the *XhoI/Sall* FRB₁ domain were subcloned into *XhoI/Sall*-digested M-F_{pk} 3-E to create M-FRB₁ and M-FRB₂. The NF- κ B-SEAP reporter plasmid was described previously.²⁵

To make the bicistronic iAkt constructs, two different internal ribosome entry sequence (IRES) elements from EMCV or poliovirus were used to link M-FRB₂ and F3- Δ PH.AKT on the same transcript. The poliovirus IRES sequence (IRESp) was *PfuI*-amplified from pTPOV-3816⁷³ with primers, 5pIRES/Mn: 5'-ata caattg ccgcgg ttc gaattc tgtttataactcccttcccgaac-3' and 3pIRES/Mun: 5'-tat caattg gtttaaac agcaaacagatagataatgagtctcac-3'. The resulting PCR products were subcloned into pCR-Blunt to create pSH5/IRESp-Mun. The 615-bp IRESp *MunI* fragment was ligated into *EcoRI*-digested pSH1/M-FRB₂-E to create pSH1/M-FRB₂-E-IRESp. Finally, the *NotI/EcoRI* F3- Δ PH.AKT fragment from pSH1/F3- Δ PH.AKT was blunt-ligated into the *PmeI* site to create pSH1/M-FRB₂-E-IRESp-F3 Δ PH.Akt, renamed as iAkt_b. The bicistronic vector iAkt_b utilizes the EMCV IRES and was made by a comparable strategy. For establishing Jurkat.iAkt cell lines, the bicistronic *NotI/MunI* fragment from iAkt_b was subcloned into *NotI/EcoRI*-digested pBJ5-neo (described previously) to create pBJ5-neo/iAkt_b.

Cell culture

293T human embryonic kidney cells (ATCC, Manassas, VA, USA) and Jurkat (ATCC), Jurkat-TAG⁷⁴ and Jurkat.iAkt were maintained in DMEM or RPMI-1640, respectively, containing 10% fetal bovine serum (FBS) and antibiotics. The Jurkat.iAkt line was derived by transfecting Jurkat cells with *NdeI*-linearized pBJ5-neo/iAkt_b plasmid followed by G418 (1 mg/ml) selection. Clones were screened by anti-HA immunoblotting.

Reagents

Etoposide, propidium iodide, RNase A and staurosporine were from Sigma (St Louis, MO, USA); wortmannin

and LY294002 were from Calbiochem (La Jolla, CA, USA); Rapalog/AP22783 was from Ariad Pharmaceuticals (Cambridge, MA, USA; www.ariad.com/regulationkits/). Anti-Fas antibody (CH.11) was obtained from Kamiya Biomedical (Thousand Oaks, CA, USA), monoclonal anti-HA (HA.11) was from BABCO (now CRP, Princeton, NJ, USA), polyclonal anti-HA was from Upstate Biotechnology (Lake Placid, NY, USA), anti-caspase-3 was from Santa-Cruz Biotechnology (Santa Cruz, CA, USA) and anti-PARP was from Roche-Boehringer-Mannheim (Indianapolis, IN, USA).

Electroporation and SEAP assay

Jurkat-TAG cells in logarithmic-phase growth were electroporated (950 μ F, 250 V; Gene Pulser II (BioRad, Hercules, CA, USA)) with expression plasmids and 2 μ g of the NF- κ B-SEAP reporter plasmid. After 24 h, transfected cells were stimulated with sub-optimal levels of the phorbol ester PMA (5 ng/ml) along with log dilutions of the heterodimerizing CID, AP22783, and additional treatments as stated in the figure legends. After an additional 24 h, supernatants were assayed for SEAP activity as described previously.²⁵

Western blots

293T cells seeded in six-well plates were transiently transfected with 2 μ g of different expression constructs in 6 μ l FuGENE6 (Boehringer-Mannheim, Indianapolis, IN, USA) in Opti-MEM-I medium for 24 h followed by serum starvation for an additional 24 h. Cells were then treated with different agents and harvested at different time-points as stated. After washing twice in ice-cold PBS, cell pellets were lysed in RIPA buffer containing protease inhibitors (CytoSignal, Irvine, CA, USA). Equal amounts of protein from each sample were separated on 10% SDS-PAGE gels and transferred to PVDF membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Phospho-specific antibodies against Akt (T308 or S473 site) (Cell Signaling, Beverly, MA, USA) were used for measuring Akt phosphorylation, and the signal was detected by AP-conjugated secondary antibodies (NEB, Beverly, MA, USA) and CDP-Star chemiluminescence reagent (NEN Life Science, Boston, MA, USA).

Immunoprecipitation and in vitro Akt kinase assay

Jurkat.iAkt_b were serum starved for 24 h followed by treatment with AP22783 or serum for 30 min. Cells were then lysed in a lysis buffer provided with the Akt Kinase Assay kit (Cell Signaling, Beverly, MA, USA), and F3- Δ PH.AKT-E was immunoprecipitated with polyclonal anti-HA antibody. Antibody-antigen complexes were washed three times in lysis buffer and once in kinase buffer. *In vitro* kinase assays for Akt were performed using a GSK3 α/β 'crosstide'. The extent of crosstide phosphorylation was determined by anti-GSK α/β immunoblotting according to the manufacturer's protocol.

Apoptosis and flow cytometry

Jurkat.iAkt were serum starved for 24 h followed by pre-treatment with AP22783 in 0, 2 or 10% FBS for 40 min. After incubation with apoptosis-inducing stimuli for the periods indicated, cells were harvested and washed twice in ice-cold PBS and fixed in 70% ethanol. Cells were stained in 50 μ g/ml propidium iodide and 100 μ g/ml RNase A for 30 min at 37°C, and hypodiploid cells were

quantitated by flow cytometry using a Beckman-Coulter EPICS XL-MCL. For determination of caspase-3 activation and PARP cleavage after staurosporine treatment, cell pellets were lysed in Laemmli sample buffer containing 5% (v/v) β -mercaptoethanol (Bio-Rad, Hercules, CA, USA), and equal amounts of protein were separated on 6 (for PARP) or 12% (for caspase3) SDS-PAGE followed by immunoblotting with anti-caspase-3 and anti-PARP antibodies.

Note added in proof

The high basal activity of F3-Akt and the observed PI-3K-independence of iAkt signaling may be partly explained by the high basal levels of phosphatidylinositides in Jurkat cells and other tumor lines, due to PTEN deficiency.⁷⁵

Acknowledgements

We thank Gerald R Crabtree and Tim Clackson for a critical reading of the manuscript and M. Neiditch, S-H Lee, and E Lowenthal for technical assistance. We thank ARIAD Pharmaceuticals for generously providing AP22783 and the FRB₁ template DNA, P Tsichlis for plasmid pCMV6-HA-Akt, M Greenberg for plasmid pCMV-HA-AktK179M, and R Lloyd for pTPOV-3816. This work was supported by NIH grants R01-CA87569 and U01-CA84296.

References

- Lander ES. Initial sequencing and analysis of the human genome. *Nature* 2001; 409: 860-921.
- Wymann MP, Pirola L. Structure and function of phosphoinositide 3-kinases. *Biochim Biophys Acta* 1998; 1436: 127-150.
- Chan TO, Rittenhouse SE, Tsichlis PN. AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu Rev Biochem* 1999; 68: 965-1014.
- Bellacosa A, Testa JR, Staal SP, Tsichlis PN. A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science* 1991; 254: 274-277.
- Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. *Genes Dev* 1999; 13: 2905-2927.
- Franke TF *et al*. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* 1995; 81: 727-736.
- Shaw M, Cohen P, Alessi DR. The activation of protein kinase B by H202 or heat shock is mediated by phosphoinositide 3-kinase and not by mitogen-activated protein kinase-activated protein kinase-2. *Biochem J* 1998; 336: 241-246.
- Zhu WZ *et al*. Dual modulation of cell survival and cell death by beta(2)-adrenergic signaling in adult mouse cardiac myocytes. *Proc Natl Acad Sci USA* 2001; 98: 1607-1612.
- Kroner C, Eybrechts K, Akkerman JW. Dual regulation of platelet protein kinase B. *J Biol Chem* 2000; 275:27790.
- Filippa N *et al*. Mechanism of protein kinase B activation by cyclic AMP-dependent protein kinase. *Mol Cell Biol* 1999; 19: 4989-5000.
- Balendran A *et al*. PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. *Curr Biol* 1999; 9: 393-404.
- Toker A, Newton AC. Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. *J Biol Chem* 2000; 275: 8271-8274.
- Meier R, Thelen M, Hemmings BA. Inactivation and dephosphorylation of protein kinase Balpha (PKBalpha) promoted by hyperosmotic stress. *EMBO J* 1998; 17: 7294-7303.
- Nakatani K *et al*. Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. *J Biol Chem* 1999; 274: 21528-21532.
- Yuan ZQ *et al*. Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. *Oncogene* 2000; 19: 2324-2330.
- Liu AX *et al*. AKT2, a member of the protein kinase B family, is activated by growth factors, v-Ha-ras, and v-src through phosphatidylinositol 3-kinase in human ovarian epithelial cancer cells. *Cancer Res* 1998; 58: 2973-2977.
- Stambolic V *et al*. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 1998; 95: 29-39.
- Li J *et al*. The PTEN/MMAC1 tumor suppressor induces cell death that is rescued by the AKT/protein kinase B oncogene. *Cancer Res* 1998; 58: 5667-5672.
- Cantley L, Neel BG. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci USA* 1999; 96: 4240-4245.
- Cross DA *et al*. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 1995; 378: 785-789.
- Gossen M *et al*. Transcriptional activation by tetracyclines in mammalian cells. *Science* 1995; 268: 1766-1769.
- Jackson P, Baltimore D, Picard D. Hormone-conditional transformation by fusion proteins of c-Abl and its transforming variants. *EMBO J* 1993; 12: 2809-2819.
- Picard D. Regulation of protein function through expression of chimaeric proteins. *Curr Opin Biotechnol* 1994; 5: 511-515.
- Samuels ML, Weber MJ, Bishop JM, McMahon M. Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human raf-1 protein kinase. *Mol Cell Biol* 1993; 13: 6241-6252.
- Spencer DM, Wandless TJ, Schreiber SL, Crabtree GR. Controlling signal transduction with synthetic ligands. *Science* 1993; 262: 1019-1024.
- Spencer DM. Creating conditional mutations in mammals. *Trends Genet* 1996; 12: 181-187.
- Spencer DM *et al*. Functional analysis of Fas signaling *in vivo* using synthetic inducers of dimerization. *Curr Biol* 1996; 6: 839-847.
- Blau CA, Peterson K, Drachman JG, Spencer DM. A proliferative switch for genetically modified cells. *Proc Natl Acad Sci USA* 1997; 94: 3076-3081.
- Luo Z *et al*. Oligomerization activates c-Raf-1 through a Ras-dependent mechanism (see comments). *Nature* 1996; 383: 181-185.
- MacCorkle RA, Freeman KW, Spencer DM. Synthetic activation of caspases: artificial death switches. *Proc Natl Acad Sci USA* 1998; 95: 3655-3660.
- Ho SN *et al*. Dimeric ligands define a role for transcriptional activation domains in reinitiation. *Nature* 1996; 382: 822-826.
- Rivera VM *et al*. A humanized system for pharmacologic control of gene expression. *Nat Med* 1996; 2: 1028-1032.
- Spencer DM *et al*. A general strategy for producing conditional alleles of Sre-like tyrosine kinases. *Proc Natl Acad Sci USA* 1995; 92: 9805-9809.
- Holsinger LJ *et al*. Signal transduction in T lymphocytes using a conditional allele of Sos. *Proc Natl Acad Sci USA* 1995; 95: 9810-9814.
- Pollock R *et al*. Delivery of a stringent dimerizer-regulated gene expression system in a single retroviral vector. *Proc Natl Acad Sci USA* 2000; 97: 13221-13226.
- Kohn AD, Takeuchi F, Roth RA. Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. *J Biol Chem* 1996; 271: 21920-21926.
- Chen J, Zheng XF, Brown EJ, Schreiber SL. Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. *Proc Natl Acad Sci USA* 1995; 92: 4947-4951.

- 38 Choi J, Chen J, Schreiber SL, Clardy J. Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. *Science* 1996; 273: 239-242.
- 39 Kane LP, Shapiro VS, Stokoe D, Weiss A. Induction of NF-kappaB by the Akt/PKB kinase. *Curr Biol* 1999; 9: 601-604.
- 40 Madrid LV *et al*. Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. *Mol Cell Biol* 2000; 20: 1626-1638.
- 41 Madrid LV, Mayo MW, Reuther JY, Baldwin Jr. AS. Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the I kappa B kinase and activation of the mitogen-activated protein kinase p38. *J Biol Chem* 2001; 276: 18934-18940.
- 42 Andjelkovic M *et al*. Role of translocation in the activation and function of protein kinase B. *J Biol Chem* 1997; 272: 31515-31524.
- 43 Alessi DR *et al*. 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. *Curr Biol* 1997; 7: 776-789.
- 44 Anderson KE, Coadwell J, Stephens LR, Hawkins PT. Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B. *Curr Biol* 1998; 8: 684-691.
- 45 Bellacosa A *et al*. Akt activation by growth factors is a multiple-step process: the role of the PH domain. *Oncogene* 1998; 17: 313-325.
- 46 Alessi DR *et al*. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B. *Curr Biol* 1997; 7: 261-269.
- 47 Tamaoki T *et al*. Staurosporine a potent inhibitor of phospholipid/Ca⁺⁺-dependent protein kinase. *Biochem Biophys Res Commun* 1986; 135: 397-402.
- 48 Secrist JP, Sehgal I, Powis G, Abraham RT. Preferential inhibition of the platelet-derived growth factor receptor tyrosine kinase by staurosporine. *J Biol Chem* 1990; 265: 20394-20400.
- 49 Rohn JL *et al*. The opposing roles of the Akt and c-Myc signaling pathways in survival from CD95-mediated apoptosis. *Oncogene* 1998; 17: 2811-2818.
- 50 Hausler P *et al*. Protection of CD95-mediated apoptosis by activation of phosphatidylinositol 3-kinase and protein kinase B. *Eur J Immunol* 1998; 28: 57-69.
- 51 Kaufmann SH. Cell death induced by topoisomerase-targeted drugs: more questions than answers. *Biochim Biophys Acta* 1998; 1400: 195-211.
- 52 Hemmings BA. Akt signaling: linking membrane events to life and death decisions. *Science* 1997; 275: 628-630.
- 53 Crabtree GR, Schreiber SL. Three-part inventions: intracellular signaling and induced proximity. *Trends Biochem Sci* 1996; 21: 418-422.
- 54 Franke TF, Kaplan DR, Cantley LC, Tokier A. Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science* 1997; 275: 665-668.
- 55 Kohn AD *et al*. Construction and characterization of a conditionally active version of the serine/threonine kinase Akt. *J Biol Chem* 1998; 273: 11937-11943.
- 56 Liberles SD, Diver ST, Austin DJ, Schreiber SL. Inducible gene expression and protein translocation using nontoxic ligands identified by a mammalian three-hybrid screen. *Proc Natl Acad Sci USA* 1997; 94: 7825-7830.
- 57 Mirza AM, Kohn AD, Roth RA, McMahon M. Oncogenic transformation of cells by a conditionally active form of the protein kinase Akt/PKB. *Cell Growth Differ* 2000; 11: 279-292.
- 58 Bordignon C *et al*. Transfer of the HSV-tk gene into donor peripheral blood lymphocytes for *in vivo* modulation of donor anti-tumor immunity after allogeneic bone marrow transplantation. *Hum Gene Ther* 1995; 6: 813-819.
- 59 Thomis DC *et al*. A Fas-based suicide switch in human T cells for the treatment of graft-versus-host disease. *Blood* 2001; 97: 1249-1257.
- 60 Linnik MD, Zahos P, Geschwind MD, Federoff HJ. Expression of bcl-2 from a defective herpes simplex virus-1 vector limits neuronal death in focal cerebral ischemia. *Stroke* 1995; 26: 1670-1674; discussion 1675.
- 61 Lawrence MS *et al*. Herpes simplex viral vectors expressing Bcl-2 are neuroprotective when delivered after a stroke. *J Cereb Blood Flow Metab* 1997; 17: 740-744.
- 62 Antonawich FJ, Federoff HJ, Davis JN. BCL-2 transduction, using a herpes simplex virus amplicon, protects hippocampal neurons from transient global ischemia. *Exp Neurol* 1999; 156: 130-137.
- 63 Shimazaki K *et al*. Adeno-associated virus vector-mediated bcl-2 gene transfer into post-ischemic gerbil brain *in vivo*: prospects for gene therapy of ischemia-induced neuronal death. *Gene Therapy* 2000; 7: 1244.
- 64 Bilbao G *et al*. Reduction of ischemia-reperfusion injury of the liver by *in vivo* adenovirus-mediated gene transfer of the anti-apoptotic Bcl-2 gene. *Ann Surg* 1999; 230: 185-193.
- 65 Matsui T *et al*. Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia *in vivo*. *Circulation* 2001; 104: 330-335.
- 66 Lu Y *et al*. Activated Akt protects the lung from oxidant-induced injury and delays death of mice. *J Exp Med* 2001; 193: 545-549.
- 67 Zheng L *et al*. Cytoprotection of human umbilical vein endothelial cells against apoptosis and CTL-mediated lysis provided by caspase-resistant Bcl-2 without alterations in growth or activation responses. *J Immunol* 2000; 164: 4665-4671.
- 68 Bilbao G *et al*. Genetic modification of liver grafts with an adenoviral vector encoding the Bcl-2 gene improves organ preservation. *Transplantation* 1999; 67: 775-783.
- 69 Bilbao G *et al*. Genetic cytoprotection of human endothelial cells during preservation time with an adenoviral vector encoding the anti-apoptotic human Bcl-2 gene. *Transplant Proc* 1999; 31: 1012-1015.
- 70 Contreras JL *et al*. Gene transfer of the Bcl-2 gene confers cytoprotection to isolated adult porcine pancreatic islets exposed to xenoreactive antibodies and complement. *Surgery* 2001; 130: 166-174.
- 71 Bellacosa A *et al*. Structure, expression and chromosomal mapping of c-akt: relationship to v-akt and its implications. *Oncogene* 1993; 8: 745-754.
- 72 Datta SR *et al*. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 1997; 91: 231-241.
- 73 Lloyd RE, Grubman MJ, Ehrenfeld E. Relationship of p220 cleavage during picornavirus infection to 2A proteinase sequencing. *J Virol* 1988; 62: 4216-4223.
- 74 Northrop JP, Ullman KS, Crabtree GR. Characterization of the nuclear and cytoplasmic components of the lymphoid-specific nuclear factor of activated T cells (NF-AT) complex. *J Biol Chem* 1993; 268: 2917-2923.
- 75 Shan X *et al*. Deficiency of PTEN in Jurkat T cells causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation. *Mol Cell Biol* 2000; 20: 6945-6957.